Ceriodaphnia dubia 96-Hour Acute Toxicity Test

1.0 OBJECTIVE

In laboratory tests designed to determine the toxicity of low-salinity water samples, *Ceriodaphnia* neonates are exposed to test solutions for 96 hours, after which the percentage mortality is determined in each toxicant concentration. Observed effects may be related to the presence of contaminants or to naturally occurring factors. In order to correctly interpret toxicity results, concentrations of chemical contaminants should be analyzed, as well as other water quality parameters, such as pH, dissolved oxygen, hardness, alkalinity, temperature, ammonia and conductivity.

In this procedure, water samples collected from field stations are divided into randomly numbered replicate test containers in the laboratory. Five *Ceriodaphnia* neonates are placed into each replicate container. Each beaker is monitored daily for mortality, and is renewed at 48 hours. After a 96-hour exposure, survival is counted and recorded to give an estimate of sample toxicity. Because the test measures effects on an early life-stage of an ecologically important species possessing relatively stringent water quality requirements, the results constitute a good basis for decisions concerning either hazard evaluation or the suitability of estuarine waters for aquatic life (EPA, 1993).

2.0 EQUIPMENT

The following equipment is necessary to conduct the toxicity test at the Marine Pollution Studies Laboratory at Granite Canyon (MPSL). The word "clean" here and throughout this procedure means that the item has been cleaned according to the MPSL glassware cleaning procedures outlined in a separate standard operating procedure (SOP).

2.1 Culture

- Pipettes, tubing, and clean air system
- 2-liter beakers or similar volume containers
- 4:1 water prepared from Nanopure® (or distilled) water and Perrier® or Evian® (25± 1°C)
- YCT and *Selenastrum* for feeding, purchased from Aquatic Biosystems (Fort Collins, CO)
- *Ceriodaphnia* neonates (<24 hours old, supplied by ToxScan (Watsonville, CA)

2.2 Test Initiation/Termination

- Environmental chamber (25± 1°C, ambient laboratory illumination for 16 hours/day)
- 50-ml clean glass beakers (5 per sample, 3 per reference toxicant dilution), with covers
- 100-ml volumetric flask for reference toxicant dilutions
- 10-ml and micropipettors and pipettes for reference toxicant dilutions
- Cupric chloride stock solution (10,000 µg/L Cu)
- Randomization sheet to arrange and identify test containers

• Data sheets
• Gloves and appropriate safety gear (see MPSL lab safety manual)
• Sample vials for reference toxicant analysis (new polyethylene 30 ml, acid washed)
• Dissecting microscope for counting neonates
• Disposable plastic pipettes with cut-off tips (for handling animals)

2.3 Water Quality

• Meters, probes, spectrophotometer, digital titrator and standards for measuring pH, dissolved oxygen, hardness, alkalinity, ammonia, and conductivity
• Thermometers (glass spirit thermometer and continuously recording thermometer)
• Graduated pipettes (10 ml) and hand pipette pump for water quality sampling
• Water quality vials (30 ml glass)
• Gloves and appropriate safety gear (see MPSL lab safety manual)

2.4 Dilution Water

In every step of this procedure, use Granite Canyon Nanopure® (or distilled) water mixed with Perrier® or Evian® in a 4:1 ratio. Conductivity should not exceed 3000 μS/cm at any time. Hardness (as $CaCO_3$) should not exceed 700 mg/liter.

3.0 EXPERIMENTAL DESIGN

Aquatic toxicity tests can be used as screening tools or as part of more comprehensive studies to assess water quality. Careful consideration must be given to site characteristics, reference site selection, field replication, choice of synoptic measures, seasonal factors, and comprehensive planning and peer review to determine that study designs are adequate to meet program objectives.

This laboratory toxicity test consists of five replicate test beakers for each sample concentration. Beakers are arranged randomly, and each receives five *Ceriodaphnia* neonates. The quality of test animals and testing conditions is determined through concurrent testing of reference toxicants (positive controls) and control water (negative controls). Testing of reference sites or receiving water is recommended to demonstrate the suitability of test sites in the absence of toxic contaminant concentrations. Test conditions of temperature and photoperiod are controlled as indicated below, and pH, NH_3, conductivity, and dissolved oxygen are measured at the beginning and end of the exposure. Temperature is measured continuously, and hardness and alkalinity are measured at the beginning of the test.

4.0 PREPARATION OF SAMPLES FOR TESTING

One day before test initiation, the volume necessary for test initiation should be placed in the constant temperature room (25°C) to allow oxygen concentrations to equilibrate below super-saturated levels; the minimum time should be 12 hours. Prepare five replicate 50-ml beakers for each sample to be tested. Consult the random number sheet to ensure proper randomization. Each container receives 15 ml of test solution.

5.0 CONTROLS

5.1 Dilution and Conductivity Controls

There should be two dilution controls: one consisting of 4:1 culture water, and another that matches the highest conductivity. If samples are diluted because of high conductivity, the high conductivity control should be prepared to reflect this dilution. Prepare the conductivity control by starting with 4:1 culture water and adding 1-µm filtered seawater dropwise until the initial conductivity is reached. Dilute back to the final conductivity with 4:1 culture water.

5.2 Reference Toxicant Tests (Positive Controls)

For cultured organisms, conduct a concurrent reference toxicant at least monthly. The reference toxicant test indicates the sensitivity of the organisms and the suitability of the test methodology.

Reagent grade cupric chloride ($CuCl_2$) should be used as the reference toxicant for *Ceriodaphnia* tests, unless another toxicant is specified by the Regional Water Quality Control Board or other appropriate regulatory agency. Prepare a 10,000 µg/l Cu stock solution by adding 0.0268 g of reagent grade $CuCl_2$ to a final volume of one liter of distilled water in a plastic volumetric flask. Cap tightly and mix thoroughly. Sample and log the reference toxicant stock solution at the beginning of the test for chemical verification of the copper concentration. Acidify samples for analysis in clean sample vials with 1% by volume 14N-reagent grade nitric acid per 30 ml of sample.

Reference toxicant solutions should be three to five replicates of 0 (control), 5.6, 10, 18, 32, and 56 µg Cu/liter. Other concentrations may be added between these if greater precision is desired for quality control chart purposes. Prepare 100 ml of each concentration by adding stock solution (see dilution schedule) to a 100-ml plastic volumetric flask and fill with culture water. Aliquot each concentration to randomly numbered test containers as indicated on the random number sheet, and into water quality vials. Start with the control solutions and progress to the highest concentration to minimize contamination. Place the reference toxicant test containers in the constant temperature room, cover, and allow to equilibrate.

All tests (sample and reference toxicant) must use neonates from the same culture. They must be handled in the same way and delivered to the test containers at the same time.

6.0 TEST ORGANISMS

6.1 Culturing *Ceriodaphnia* and Isolating Neonates

The water flea, *Ceriodaphnia dubia*, occurs in littoral areas of lakes, ponds, and marshes throughout most of the world. *Ceriodaphnia* sensitivity to contaminants and their ease of laboratory culture make them suitable organisms for determining the toxicity of chemical compounds, complex effluents, and fresh waters. *Ceriodaphnia* are supplied in one or two small jars. These should be emptied into a 1-liter glass beaker and placed in the environmental chamber upon arrival. Neonates should be fed 1 ml each of *Selenastrum* and YCT upon arrival.

6.2 Neonate Loading

Pour a subsample of *Ceriodaphnia* neonates from the glass beaker to a crystallizing dish. Using a trimmed plastic disposable pipette, transfer one to two neonates at a time into the test containers. Continue until each container has 5 animals. Maintain water temperature (25°C ± 1) by sorting animals in the constant temperature room where the test is being held. Once loaded, all test containers should be covered with a clear plastic sheet.

7.0 MONITORING THE TOXICITY TEST

7.1 Counting *Ceriodaphnia* Mortality

Test duration is 96 hours. Check all test containers daily, and record the number of live animals. Also count the number dead to ensure that the total number of animals in the container at the start of the test was 5; if not, record this on the data sheet. Immobile *Ceriodaphnia* that do not respond to a stimulus are considered dead. The stimulus should be a gentle stream of water from a disposable pipette. *Ceriodaphnia* that exhibit any response visible under the dissecting scope are considered living. Remove dead animals.

7.2 Measuring Water Quality in Test Containers

Measure temperature, dissolved oxygen, pH, ammonia, and conductivity at the beginning and end of the test. Hardness and alkalinity should be measured at the beginning of the test. Sample the initial test solutions at the time of dilution preparation. Water quality should also be measured on old and new dilutions at the time of renewal. Renewal water quality parameters include dissolved oxygen, pH, conductivity, and temperature.

Water quality should be measured only initially on reference toxicant tests.

8.0 TEST SOLUTION RENEWAL

The test duration is 96 hours. Because toxicity may change over short time periods in test containers, the test solutions must be renewed after 48 hours. Prepare new test solutions as in section 6.3. One day before solution renewal, the volume necessary for renewal should be placed in the constant temperature room (25°C) to allow oxygen concentrations to equilibrate below super-saturated levels; the minimum time should be 12 hours. These samples must be aerated if dissolved oxygen concentrations exceed maximum values allowed.

Two hours prior to the renewal, feed the existing test beakers 200 µl 3:1 *Selenastrum* and YCT. After two hours, use a dissecting microscope and a 10-ml pipette to remove 7.5 ml of solution from each test container, and replace it with fresh solution. Use care to not remove the neonates in the process.

9.0 TERMINATING THE TOXICITY TEST

After 96 hours of exposure, final mortality counts are made.

Final water quality must be sampled at the termination of the test. Deliver a sample from each site into pre-labeled water quality containers. Measure and record temperature, dissolved oxygen, pH and conductivity of each sample. Only measure temperature for the reference toxicant test.

Take the completed data sheet to the office for data entry and analysis. Notify the data analyst that the data has arrived. Make sure the data sheets are placed in the proper location and that the person keeping track of the data knows where it is.

10.0 DATA HANDLING AND TEST ACCEPTABILITY

Immediately after test termination, check the data sheet to determine whether dilution water and conductivity controls have acceptable survival (mean of 90% or greater). If not, notify the project officer without delay.

Tests with temperature, salinity, or dissolved oxygen measurements outside the specified ranges, may be considered conditionally acceptable based on the project officer's best professional judgment. Acceptable temperatures are 25 ± 1°C; acceptable dissolved oxygen concentration is 60-100% saturation.

11.0 REFERENCES

US EPA. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. U.S. Environmental Protection Agency, Office of Research and Development. EPA/600/4-90/027F. August 1993.

12.0 TEST SUMMARY

Species:	*Ceriodaphnia dubia*
Test Duration:	96 hours
Endpoint:	Survival
Renewals:	One at 48 hours
Organism Source	Toxscan (Watsonville, CA)
Age of Test Organisms:	<24 hours
Test Conductivity:	<3000 μS
Test Temperature:	25 ± 1°C
Dilution Water:	Evian®:Nanopure® 4:1
Light intensity:	Ambient laboratory illumination (10-20 μE/m^2/s)
Photoperiod:	16 hour light: 8 hour dark
Replication:	5 (samples), 3 (reference toxicant)
Test Containers:	50-ml glass beakers
Test Solution Volume:	15 ml
Loading:	5 animals per beaker
Feeding:	In culture prior to test initiation and 200 μl 3:1 Selenastrum:YCT 2 hours before renewal
Water Quality:	pH, dissolved oxygen, temperature, conductivity, NH_3 (beginning, old/new renewal, end); hardness, alkalinity (beginning)
Reference Toxicant:	copper chloride ($CuCl_2$)
Daily Monitoring:	count alive and remove dead
Acceptability Criteria:	mean survival in dilution water controls at least 90%